

## Evaluation of the Therapeutic Effect of Radioattenuated Yeast Cells in Experimental Paracoccidioidomycosis

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### Abstract

*Paracoccidioides brasiliensis* is the agent of *paracoccidioidomycosis* (PCM), the most prevalent deep mycosis in Latin America. The treatment varies according to the chemotherapeutic drug and the disease severity, and the lack of it can result in high frequency of relapse and sequels. Thus, the search for new therapeutic alternatives is necessary. The aim of this study was to evaluate the therapeutic effect of *P. brasiliensis* yeast cells attenuated by gamma irradiation (LevRad) in Balb/c mice. Mice immunized with LevRad and/or treated with fluconazole presented a significant decrease in the CFU recovery from the lung, liver and spleen in comparison with non-immunized and non-treated infected mice (60 and 120 days after infection). After 120 days from fungal inoculation, no fungi colonies were obtained from the organs of treated and immunized mice. The tissue structure of these organs was largely preserved. At the same time, anti-Mexo specific IgG antibodies levels and TGF- $\beta$ , IFN- $\gamma$ , iNOS transcript levels were high and a decrease on the IL-4, IL-10 and TNF- $\alpha$  transcript levels was also verified. An additive protective effect of immunization with LevRad associated to chemotherapy in a PCM experimental model was verified, providing evidence of the therapeutic effect of attenuated yeast for fungal infections.

**Keywords:** Therapeutic vaccine; *Paracoccidioides brasiliensis*; Gamma irradiation; *Paracoccidioidomycosis*

### Abbreviations:

PCM: *Paracoccidioidomycosis*; Pb18: *Paracoccidioides brasiliensis* strain; LevRad: *P. brasiliensis* yeast cells attenuated by gamma irradiation; CETEA: Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais; CFU: Colony Forming Units; TMB: Tetramethylbenzidine

### Introduction

The most prevalent deep mycosis in Latin America, *Paracoccidioidomycosis* (PCM), is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. It is estimated that approximately 10 million people are infected, although most of them are asymptomatic [1]. A total of 3181 lethal PCM cases were registered in Brazil between the years 1980 and 1995. However, it is plausible to believe that this number is still underestimated, since the disease is not indicated for mandatory reporting [2].

PCM may manifest in two distinct forms: the chronic (adults) and the acute/subacute (adults and juveniles). The acute form affects the reticulo-endothelial system and it may be fatal, whereas the chronic form often causes lesions in the lungs with consequent dissemination to other organs and tissues. Such damage may result in severe restriction of respiratory function. This medical situation might have

negative effects on the patients' working performance, which strongly affects their life quality with significant social and economic factors [3,4].

The therapy for PCM consists on the use sulfonamides, polyenes (amphotericin B) and azoles derivatives. The lack of treatment is usually fatal [5]. Normally, the therapy consists in a long-term treatment, which may last from 2 to 6 months. However, long term drug administration enhances the chances of treatment failure due to occasional interruption by the patient. Such interruption may increase the frequency of reactivation of quiescent focus. Therefore, developing new and more effective chemotherapeutic strategies are of great importance to better the immune protective response and to help controlling the disease [6,7].

There are some reports describing the role of antigens inducing immune responses against *P. brasiliensis* such as soluble antigen, purified antigen, peptide, DNA vaccine and the use of attenuated yeast cells [8-22].

Live-attenuated pathogens have been used successfully as vaccine candidates against various viral and bacterial pathogens [23]. There is no fungal vaccine available at present. Our group have been investigated the potential of *P. brasiliensis* yeast cells attenuated by gamma irradiation (LevRad) to induce protection against a *P. brasiliensis* infection. The endovenous immunization of Balb/c mice with the radioattenuated yeast cells (without the use of adjuvant), was able to elicit a potent and durable protection against infective highly yeasts of *P. brasiliensis* [17,18].

A main concern with live-attenuated vaccines is the risk of virulence reversion. However, gamma irradiation seems to have been a viable strategy to attenuate microorganism since alterations in the nucleus and an extensive DNA fragmentation were observed [16]. Furthermore, the loss of infectivity was confirmed in immunocompetent Balb/c mice [18] as well as in immunosuppressed mice as will be shown in this work.

In this study, the immunotherapeutic potential of LevRad, in association or not with fluconazole was tested to assess the concept that radioattenuated yeast could be a useful therapeutic tool for PCM or other fungal diseases of medical and veterinary relevance.

## Materials and Methods

### Animals

This work was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG), protocol 132/2006. Male Balb/c mice (6-8 weeks old) were purchased from UFMG (Belo Horizonte, MG, Brazil) and maintained under specific-pathogen-free conditions as previously described [24]. Balb/c mice were immunodepressed by gamma irradiation (Balb/c\*) using uniform doses of 2.5 Gy (Gray) from a uniform source of <sup>60</sup>Co gamma rays. Athymic mice (Nude), 5-8 weeks old, were kindly provided by Professor Leda Quercia Vieira from the Biologic Science Institute at the Federal University of Minas Gerais (ICB/UFMG).

### *P. brasiliensis* strain

*P. brasiliensis* yeast cells (Pb18) were maintained in YPD agar medium (0.5% yeast extract, 0.5% peptone, 1.5% D-glucose, 1.5% agar, pH 7.0) at 35°C and used after seven days of incubation. The viability of fungal suspensions was determined by staining according to Janus Green B vital dye method (Merck, Rahway, New Jersey) [25]. The viability was always higher than 90%. The virulence of the Pb18 isolate was verified by intratracheal infection of Balb/c mice and further recovering of yeast cells from their organs.

### *P. brasiliensis* attenuation

Cultures of *P. brasiliensis* yeast cells (Pb18), incubated in YPD agar medium were irradiated in the presence of oxygen and at room temperature. The irradiation was performed in a uniform source of <sup>60</sup>Co gamma rays at dose rate of 1000 Gy/h. A dose of 6.5 kGy was used to achieve attenuation as in Demichelli and colleagues [16]. The viability of irradiated fungal suspensions was determined by Janus Green B vital dye method [25] (Merck, Rahway, New Jersey). Radioattenuated *P. brasiliensis* viable yeast cells were named LevRad.

### Evaluating of LevRad infectivity

The LevRad infectivity loss was evaluated on athymic (Nude), immunodepressed (Balb/c\*) and normal (Balb/c) mice. They were inoculated by intratracheal or endovenous route ( $1 \times 10^7$  viable yeast cells of LevRad). After 30 and 90 days their organs were collected for the recovery of colony forming units (CFU) and histopathological changes. Uninfected animal (C-) and infected with non-irradiated virulent yeast cells of *P. brasiliensis* (C+) were used as control.

## Therapeutic vaccination experimental design

Male Balb/c mice were distributed in five groups (with 5 animals each): uninfected (C-), infected with non-irradiated virulent yeast cells of *P. brasiliensis* (C+), infected and treated with fluconazole (InMed), infected and immunized with LevRad (InRad) and infected and treated with fluconazole associated with LevRad (InRadMed).

The animals were infected with viable non-irradiated virulent yeast cells of *P. brasiliensis* (Pb18). Thirty days after infection, mice were immunized with LevRad or treated with fluconazole associated or not with LevRad. After that, the animals were euthanized 60 and 120 days after infection (30 and 90 days after the beginning of the treatment) and their organs were removed to isolate the colony-forming units (CFU) 4, observe histopathological changes<sup>5</sup>, and quantify iNOS enzyme and cytokine (IFN- $\gamma$ , IL-4, IL-10, TNF- $\alpha$  and TGF- $\beta$ ) transcript levels<sup>6</sup>. Sera were collected just before infection (P0) and after infection, at 30 (P30), 60 (P60) and 120 (P120) days. This procedure was performed to evaluate the immunization effectiveness through total IgG levels<sup>7</sup>. IgG1, IgG2a, IgG2b and IgG3 levels were also tested 60 and 120 days after infection<sup>8</sup>.

### Mice intratracheal infection

Male Balb/c mice were previously anesthetized by intramuscular (i.m) injection with 40  $\mu$ L of a solution containing 57% of ketamine at 80 mg/Kg (Dopalen, Vetbrands, Brazil) and 43% of xylazine at 15 mg/Kg (Dopaser, Laboratório Calier do Brasil LTDA, Brazil). After anesthesia, their necks were hyper-extended. A suspension of  $3 \times 10^5$  viable yeast cells of virulent *P. brasiliensis* (Pb 18), in 50  $\mu$ L of sterile phosphate-buffered saline solution (PBS, pH 7.2), was injected with a 30-gauge needle into the exposed trachea. The incisions were sutured with 4-0 silk.

### Infected mice immunized with LevRad

After 30 days of infection, Balb/c mice were immunized twice (at 2 week intervals), by the ocular plexus with the injection of 105 radioattenuated yeast cells in 50  $\mu$ L of sterile PBS, without adjuvant.

### Chemotherapy of infected mice

After 30 days of infection, Balb/c mice were treated intraperitoneally (for 30 days) with daily fluconazole doses at 10 mg/Kg (Mantena laboratories Hyderabad, India).

### CFU recovery in organs of infected mice immunized with LevRad and/or treated with fluconazole

CFU were isolated at 60 and 120 days after infection from the lungs, spleens and livers. The organs were removed, weighed, homogenized in PBS pH 7.2 and plated on solid brain heart infusion medium (BHI agar) (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with 4% fetal calf serum and 5% *P. brasiliensis* spent culture medium (Pb18) as growth factor. Gentamycin was added at 40 mg/l. The plates were incubated at 35°C and CFU were counted after 20 days. The results were expressed as the number of log<sub>10</sub> CFU of viable yeast cells of *P. brasiliensis* per gram of tissue per mouse in each experimental group.

## Histopathology

Balb/c mice were euthanized 60 and 120 days after infection. The lung, spleen and liver were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin & eosin (H&E) to characterize the inflammatory response or Grocott's methenamine-silver nitrate (Grocott) for identification of fungi and examined microscopically.

## iNOS enzyme and cytokines transcript levels

Real-time PCR assays were performed to quantify iNOS enzyme and cytokines transcripts levels in the lung from intratracheally infected mice submitted to the immunization with LevRad associated or not with fluconazole. The lungs were then excised from mice, mixed with Trizol LS Reagent (Invitrogen, Carlsbad, CA) and frozen at  $-70^{\circ}\text{C}$ . Total RNA was extracted according to the manufacturer's instructions. Isolated RNA was incubated with 1 U/ $\mu\text{g}$  RNA of RNase free DNase (Promega, São Paulo, SP, Brazil) for 30 min at  $37^{\circ}\text{C}$ . The samples were then purified with phenol-chloroform extraction followed by an ethanol precipitation (Sigma, St. Louis, MO, USA) and reverse transcription with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Molecular Biology Products, Pittsburgh PA, USA). Real-time quantitative PCR was carried out with 10  $\mu\text{l}$  of SYBR green PCR master mix (Applied Biosystems, São Paulo, SP, Brazil), 4.0  $\mu\text{l}$  of cDNA, and primers at a final concentration of 5 pmol, in a final volume of 20  $\mu\text{l}$ . Primers of IL-4, IL-10, TNF- $\alpha$ , TGF- $\beta$ , IFN- $\gamma$  and iNOS were designed according to Giulietti and colleagues [26] using the Primer Express® software v2.0 (Applied Biosystems, São Paulo, SP, Brazil). Samples were first submitted to the temperatures of  $50^{\circ}\text{C}$  for 2 min and  $60^{\circ}\text{C}$  for 1 min and then subjected to 45 cycles of amplification ( $95^{\circ}\text{C}$  for 15s followed by  $60^{\circ}\text{C}$  for 1.0 min) using an ABI PRISM 7900 apparatus (Applied Biosystems, São Paulo, SP, Brazil). Dissociation curves occurred at  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 15 s. All quantifications were normalized to the beta-actin housekeeping gene. A nontemplate control with no genetic material was included to verify contamination. Cytokines and iNOS enzyme transcripts levels were assessed through the comparative Ct method, using uninfected mice as the calibrator and reporter. A 2 fold increase was determined as the baseline to differentiate the gene expressed from the calibrator.

## Antibody response to LevRad

ELISA Maxisorb™ surface plates (NUNCTM Brand Products, Roskilde, Denmark) were coated overnight at  $4^{\circ}\text{C}$  with 0.5  $\mu\text{g}$  per well of *P. brasiliensis* exoantigen (Mexo) [27] in carbonate/bicarbonate buffer, pH 9.6. Wells were blocked for 1h at  $37^{\circ}\text{C}$  with 1.6% casein PBS solution, pH 7.2. The sera were collected just before (P0), as well as at 30 (P30), 60 (P60) and 120 (P120) days after infection. After that they were incubated at dilution 1:100 in 0.25% casein PBS solution, pH 7.2 for 1h at  $37^{\circ}\text{C}$ . Washes were performed in the intervals of incubations with 0.1% Tween-20 PBS solution, pH 7.2. The reaction was quantified with peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 antibodies (Southern Biotechnology Associates Inc.) diluted at 1:5000 and incubated for 1 h at  $37^{\circ}\text{C}$ . After additional washes, peroxidase activity was assayed with 100  $\mu\text{l}$  of Tetramethylbenzidine (TMB) substrates solution (Thermo Scientific Pierce, Pittsburgh PA, USA). Color development was stopped with 50  $\mu\text{l}$  of 2 N  $\text{H}_2\text{SO}_4$ . The optical density at 450 nm was measured with an automated ELISA reader (ELX 800 BIO-TEK Instruments Inc).

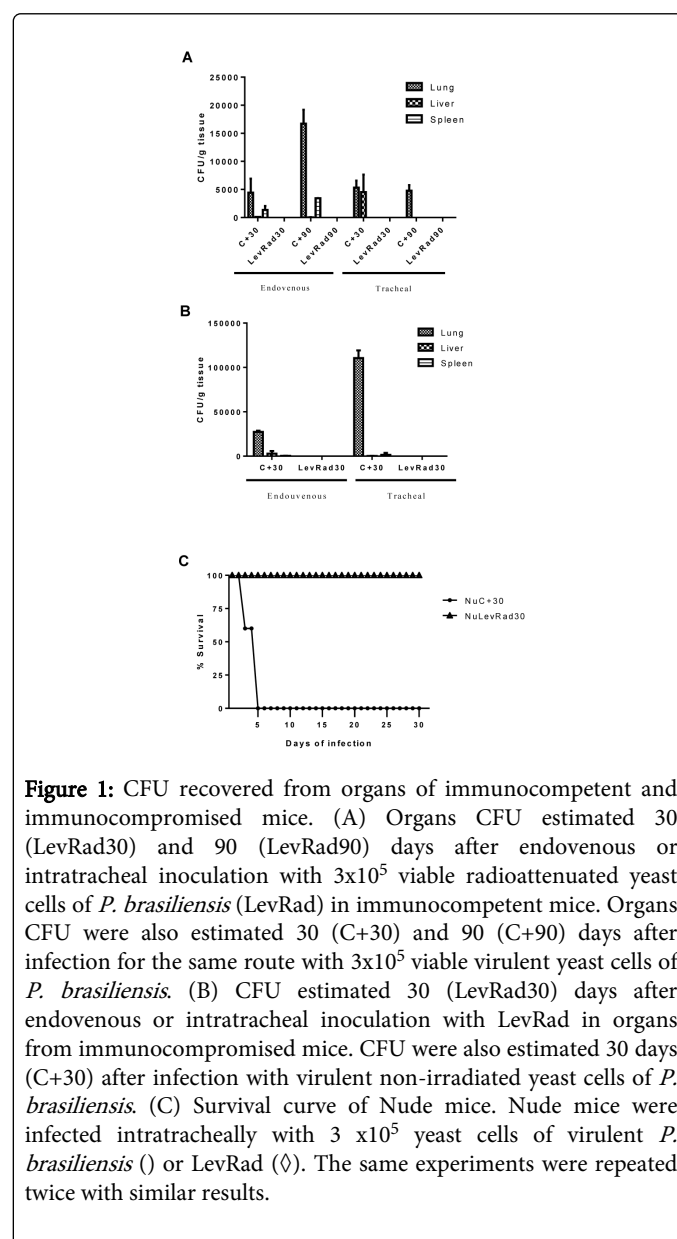
## Statistical analysis

Data were analyzed through the one-way ANOVA followed by Bonferroni test. These analyses were performed using the GraphPad Prism software package, with level of significance set at  $p < 0.05$ .

## Results

### CFU recovery from immunocompetent and immunocompromised mice inoculated with LevRad

No CFU were recovered from the organs and no histopathological changes (data not shown) were observed in the organs obtained from immunocompetent (Balb/c) and immunocompromised (Balb/c\*) mice collected 30 and 90 days after endovenous or intratracheal inoculation with LevRad (Figure 1A and 1B).



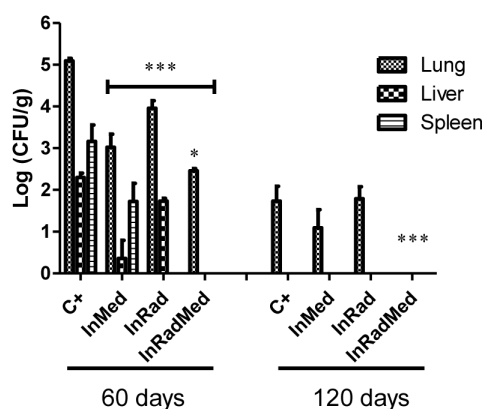
**Figure 1:** CFU recovered from organs of immunocompetent and immunocompromised mice. (A) Organs CFU estimated 30 (LevRad30) and 90 (LevRad90) days after endovenous or intratracheal inoculation with  $3 \times 10^5$  viable radioattenuated yeast cells of *P. brasiliensis* (LevRad) in immunocompetent mice. Organs CFU were also estimated 30 (C+30) and 90 (C+90) days after infection for the same route with  $3 \times 10^5$  viable virulent yeast cells of *P. brasiliensis*. (B) CFU estimated 30 (LevRad30) days after endovenous or intratracheal inoculation with LevRad in organs from immunocompromised mice. CFU were also estimated 30 days (C+30) after infection with virulent non-irradiated yeast cells of *P. brasiliensis*. (C) Survival curve of Nude mice. Nude mice were infected intratracheally with  $3 \times 10^5$  yeast cells of virulent *P. brasiliensis* (C+30) or LevRad (NuLevRad30). The same experiments were repeated twice with similar results.

Nude mice infected with the non-irradiated yeast cells (NuC+) started to die after the first day of infection and 100% of mortality was achieved at the fifth day. Nude mice inoculated with LevRad remained alive for 30 days (Figure 1C).

These results showed the LevRad inability to produce a progressive infection even in immunocompromised animals.

### CFU recovery and histopathological changes from intratracheally infected mice immunized with LevRad associated or not to fluconazole

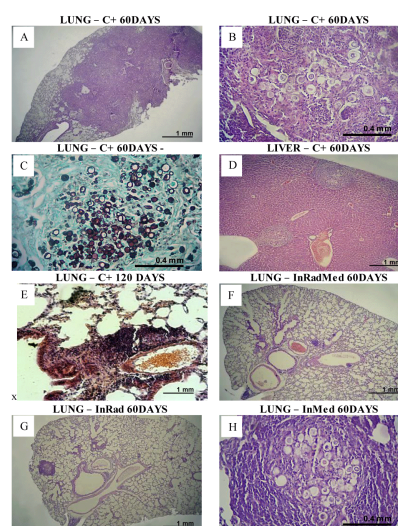
To evaluate the therapeutic effect induced by LevRad treated or/and immunized Balb/c mice organs were analyzed 60 and 120 days post infection (30 and 90 days after initiating treatment). A significant reduction of CFU levels in spleen, liver and lung was obtained 60 days after infection in the InMed, InRad and InRadMed groups in relation to C+. The only exception occurred in the InMed group, which did not present a decrease of CFU in the spleen. Moreover, CFU levels decreased significantly in the lung of InRadMed in relation to InMed and InRad. No colonies were recovered from the liver and spleen in this group. Regulation of infection occurred in 120 days in all groups (InMed and InRad, including C+), with fungal colonies only being isolated from the lungs. The best result was obtained in the group submitted to chemotherapy using LevRad combined with fluconazole (InRadMed). No colonies (CFU) were obtained from any organs (lung, liver and spleen) in this group (Figure 2).



**Figure 2:** CFU recovery from organs of treated and infected mice. The organs CFU were estimated 60 and 120 days after infection (30 and 90 days after initiating treatment). Mice were infected intratracheally with  $3 \times 10^5$  yeast cells of *P. brasiliensis*. Negative control (C-) uninfected mice; positive control (C+) infected mice with virulent non-irradiated yeast cells of *P. brasiliensis*; infected mice treated with fluconazole (InMed); infected mice treated with LevRad (InRad); infected mice treated with LevRad associated with fluconazole (InRadMed). The same experiments were repeated twice with similar results. Data are reported as Log<sub>10</sub> (CFU/g) mean+standard deviations in each experimental group (n=5). \*\*\* significant (p<0.001) difference in relation to positive control. \* significant (p<0.05) difference in relation InMed and InRad.

Furthermore, the histopathological analysis confirmed these results. After 60 days, the lungs of infected animals (C+) presented extensive tissue destruction with development of intense coalescent multifocal

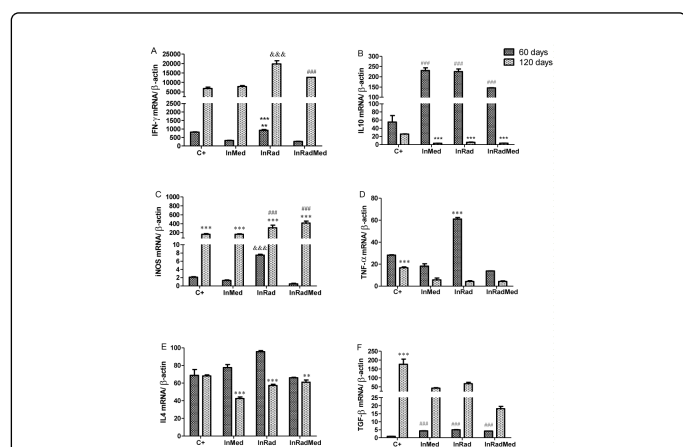
interstitial granulomatous pneumonia (Figure 3A). Neutrophilic lymphohistiocytic infiltrate associated with giant cells was observed. There was a large number of intra and extracellular fungal structures with birefringent membrane. Such morphologic feature is characteristic of *P. brasiliensis* (Figure 3B and 3C). The liver showed mild to moderate multifocal granulomas surrounded by fibrous connective tissue constituted by lymphocytes and fungal cells inside (Figure 3D). There was no histopathological alteration in the spleen (data not shown). A decrease on size of lesion was observed in 120 days in this group. A discrete focal granulomatous lesion was observed in the lung. This lesion was formed by lymphohistiocytic infiltrate with giant cells and a few numbers of viable fungal cells (Figure 3E). At the same time, the lung obtained from the InRadMed group did not present yeast cells, inflammatory infiltrated or granulomatous lesion (Figure 3F). These results were compared with the results obtained from the uninfected mice (C-). However, the lungs obtained from the InRad and InMed group (analyzed 60 days after infection) presented well organized discrete focal granulomatous lesions with low counting of yeast cells (Figure 3G and 3H). No significant injury was observed in the liver and spleen after 60 days of infection. No histopathological alterations in the liver or the spleen were observed in the studied animals after 120 days (data not shown).



**Figure 3:** Histopathology of lungs and livers from intratracheally infected mice submitted to the immunization with LevRad associated or not with fluconazole. The extension and distribution of granulomas as well as the fungal burden were observed 60 and 120 days after infection (30 and 90 days after initiating treatment). (A) intense coalescent multifocal interstitial granulomatous pneumonia (HE, 40X magnification); (B) (C) intra and extracellular fungal structures with birefringent membrane characteristic of *P. brasiliensis* (HE, 400X magnification); (D) mild to moderate multifocal granulomas surrounded by fibrous connective tissue constituted (HE, 100X magnification); (E) discrete focal granulomatous lesion (HE, 40X magnification); (F) lung free from board fungi, inflammatory infiltrated and granulomatous lesion (HE, 40X magnification); (G) well organized discrete focal granulomatous lesions (HE, 40X magnification); (H) fungal structures inside the granulomas (HE, 400X magnification).

### iNOS enzyme and cytokines transcript levels in infected mice immunized with LevRad associated or not with fluconazole

Cytokines (IFN- $\gamma$ , IL-10, TNF- $\alpha$ , TGF- $\beta$  and IL-4) and iNOS enzyme transcript levels were measured in the lung of infected mice subjected to immunization with LevRad (associated and not associated with fluconazole). One uninfected group was evaluated for basal levels of cytokines (C-), a control group of infected with non-irradiated virulent yeast cells of *P. brasiliensis* (C+), infected and treated with fluconazole (InMed), infected and treated with LevRad (InRad) and infected and treated with fluconazole associated with LevRad (InRadMed) were also examined.



**Figure 4:** Cytokine transcript levels from mice infected and treated; infected only mice (C+); infected mice treated with fluconazole (InMed); infected mice treated with LevRad (InRad); infected mice treated with LevRad associated with fluconazole (InRadMed). The same experiments were repeated twice with similar results. Data was reported as mean+standard deviations of relative expression of iNOS enzyme and cytokine in relation to  $\beta$ -actin from the lungs of 5 animals per group. IFN-  $\gamma$  (A) \*\* significant ( $p < 0.05$ ) difference related to C+; \*\*\* significant ( $p < 0.05$ ) difference for InMed and InRadMed; &&& significant ( $p < 0.05$ ) difference related to C+ and InMed; ### significant ( $p < 0.05$ ) difference of InRadMed in relation with C+ and InMed. IL-10 (B) #### and \*\*\* significant ( $p < 0.05$ ) difference in relation to C+. iNOS (C) &&& significant ( $p < 0.001$ ) difference in relation to C+, InMed and InRadMed; \*\*\* significant ( $p < 0.001$ ) difference from the first (60 days) to the second (120 days) data reading; ### significant ( $p < 0.05$ ) difference in relation to C+ and InMed. TNF-alpha (D) \*\*\* significant ( $p < 0.05$ ) difference to C+, InMed, and InRadMed; ### significant ( $p < 0.05$ ) different in C+ in relation to InMed, InRad and InRadMed. IL-4 (E) \*\*\* significant ( $p < 0.05$ ) difference between InMed and InRad; \*\* significant ( $p < 0.05$ ) difference between InRadMed and C+ ( $p < 0.05$ ). TGF-beta (F) \*\*\* e ### significant ( $p < 0.05$ ) difference between InMed, InRad and InRadMed.

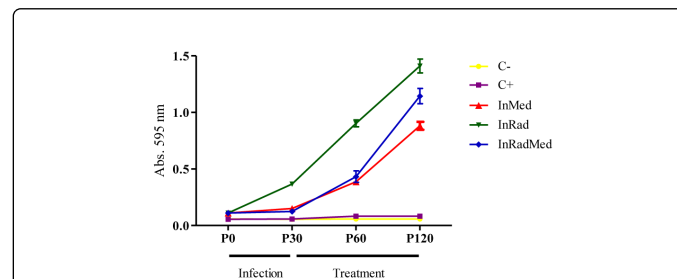
The InRad group presented a significant increase in IFN- $\gamma$  transcript levels compared with the C+, InMed and InRadMed at 60 days post infection. An increase on IFN- $\gamma$  transcript levels was observed at 120 days post infection in all groups analyzed. However, the InRad presented significant expression in relation C+, InMed and InRadMed. The IFN- $\gamma$  expression in InRadMed group also was significant compared to C+ and InMed (Figure 4A). It was possible to observe a significant increase in the IL-10 transcript levels occurring

after 60 days of infection in the InMed, InRad and InRadMed groups (in comparison with the C+ group). However, a significant decrease of these levels was observed after 120 days (Figure 4B). A significant iNOS expression occurred in the InRad groups compared to the C+ and InMed and InRadMed after 60 days of infection. All groups submitted to treatment presented an increase on the iNOS enzyme transcript levels from 60 days to 120 days post infection. At the same time, the expression in InRad and InRadMed was significant in relation to C+ and InMed (Figure 4C).

TNF- $\alpha$  transcript levels were significantly high in InRad group after 60 days of infection compared to the C+, InMed and InRadMed groups. However, at 120 days, these levels decreased in relation to C+, reaching values similar to the levels observed in the InMed and InRadMed groups (Figure 4D). At the same time, a decrease in the IL-4 transcript levels was also observed in all the tested groups, when compared to the levels registered in C+ (Figure 4E). Low TGF- $\beta$  transcript levels were observed in all groups at 60 days post infection. However, these levels increased at 120 days later. The TGF- $\beta$  transcript levels in C+ were significantly higher than in the other groups (Figure 4F).

### Antibody profile in infected mice immunized with LevRad associated or not to fluconazole

Serum was collected from treated mice for evaluation of specific responses to Mexo antigen by ELISA. After two rounds of vaccination, significant levels of anti-mexo IgG were detected in the sera of infected mice immunized with LevRad, treated and not treated with fluconazole. The animals infected with *P. brasiliensis* (C+) did not present significant anti-Mexo antibody levels. The same result was observed in the uninfected group (C-) (Figure 5). Serum of InRad groups had greater specific response than InMed group.



**Figure 5:** IgG production in serum from infected mice immunized with LevRad associated or not to fluconazole. Antibody responses against Mexo were determined just before (P0), as well as at 30 (P30), 60 (P60) and 120 (P120) days after infection by ELISA. Negative control (C-) uninfected mice; positive control (C+) infected mice with virulent non-irradiated yeast cells of *P. brasiliensis*; infected mice treated with fluconazole (InMed); infected mice treated with LevRad (InRad); infected mice treated with LevRad associated with fluconazole (InRadMed). The same experiments were repeated twice with similar results. Data were reported as mean + standard deviations of optical density (450 nm) at 1:100 serum dilution in each experimental group (n=5).

Production of anti-Mexo IgG1 isotype was verified after 60 days of infection in the sera of all animals (InMed, InRad, InRadMed and C+). This production was significant when compared to the data obtained from the C-, as well as to the other isotypes (IgG2a, IgG2b and IgG3).

However, the IgG1 levels did not present a significant difference in relation with the levels of IgG2a and IgG2b in the InRad group, as well as with the levels of IgG2b in the InRadMed group (Figure 6A). At 120 days, no alterations in the IgG1 levels were observed in comparison with the levels observed 60 days post infection. However, a significant increase on the IgG2a levels was observed in InMed, InRadMed and C+ in comparison with C-. This significant increase was also observed for IgG2b in InMed and C+ compared to C- (Figure 6B).

## Discussion

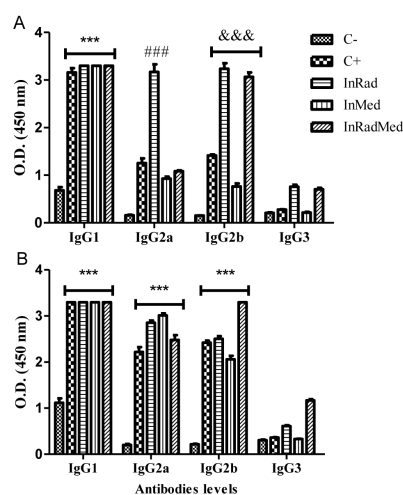
The incidence of diseases caused by pathogenic and opportunistic fungi has been increasing in the past years. The development of new immunological tools to integrate or replace non-effective and less-effective therapies is becoming more urgent nowadays.

The treatment of PCM is based on the prescription of sulfonamides, polyenes, (amphotericin B) and azoles derivatives. However, this treatment is extremely dependent on the drugs' availability and infection severity. PCM requires a long-term treatment and it is limited by toxicity of substances being used and their high cost. Moreover, resistant strains have already been reported [3,6,7,28]. It is important to highlight that the use of drugs does not guarantee the complete elimination of the fungus, which may result on reactivation of quiescent foci [5].

There are some reports describing the role of immunogenic antigens on the induction of immune responses to fungal infections, particularly PCM. Studies with yeast cells of *P. brasiliensis*, attenuated by gamma irradiation (LevRad), have been providing promising results. These yeast cells may promote a higher protection when used as a preventive vaccine, against PCM [18,19]. There are several examples of highly effective vaccines derived from live attenuated infectious agents (mainly viruses), such as, polio, measles, mumps, rubella, varicella, influenza and rotavirus [29]. For bacteria, the live attenuated Bacille Calmette-Guérin (BCG) vaccine for tuberculosis, is widely used and remained the gold standard despite efforts for the development of other vaccine formulations for this disease [30]. However, reports of vaccines preventing medically important fungal infections in humans are scarce. The only known report is related to formaldehyde-killed *Coccidioides immitis* spherules, which were tested against coccidioidomycosis [31]. However, the use of this vaccine was not possible, since wrong dosages caused toxic manifestations [32]. A vaccine based on attenuated fungi against ringworm caused by *Trichophyton verrucosum* has been successfully used in veterinary medicine [33]. A recombinant attenuated strain of *Blastomyces dermatitidis* has been promising as a potential vaccine against blastomycosis experimental [34,35]. Currently, the safety, tolerability and immunogenicity of this live-attenuated vaccine have been tested in dogs [36].

The high complexity of eukaryotic systems, associated with the high degree of variability within the *P. brasiliensis* group, may pose limitations to the success of vaccines composed by single antigens. This limitation is mainly due to the fact that only few fungi have a single immunodominant epitope possible to be targeted. Some vaccines may only reach considerable effectiveness through the combination of defined antigens promoting a coordinated interaction of many constituents involved in the host's immune response. In this context, the LevRad therapy could be a promising candidate against PCM or an experimental model for the development of new approaches concerning the prevention or treatment of other important

fungal infections. It is usual that the living agent induces strong, broad response involving multiple arms of the immune response, which provides natural immunity to disease [6] without the risk of a progressive infection. Although, it is important that the mechanisms which promote the attenuation of fungi are stable.



**Figure 6:** IgG isotypes production against Mexo in serum from infected mice immunized with LevRad associated or not to fluconazole. Antibody responses against Mexo were determined 60 (A) and 120 (B) days after infection (30 and 90 days after initiating treatment) by ELISA. Negative control (C-) uninfected mice; positive control (C+) infected mice with virulent non-irradiated yeast cells of *P. brasiliensis*; infected mice treated with fluconazole (InMed); infected mice treated with LevRad (InRad); infected mice treated with LevRad associated to fluconazole (InRadMed). The same experiments were repeated twice with similar results. Data were reported as mean + standard deviations of optical density (450 nm) at 1:100 serum dilution in each experimental group (n=5). \*\*\* significant (p<0.001) difference in relation to C- and the other isotypes (IgG2a, IgG2b and IgG3); ### significant (p<0.001) difference in relation to C-, C+, InMed and InRadMed; &&& Significant (p<0.001) difference in relation to C- and C+ and InMed.

Previous studies have already reported that radioattenuated fungi suffer an extensive DNA fragmentation, beyond cell repairment mechanisms. This leads to an irreversible loss of yeast reproductive ability and virulence [16,19,33]. The inability of LevRad to cause infection was confirmed in this study using immunocompromised (BALB/c\*) and immunocompetent (BALB/c) mice. No CFU or histopathological alterations were observed, 30 and 90 days after inoculation with LevRad, in the organs of the studied animals. Athymic mice (Nude) achieved 100% of mortality at the fifth day of experiment, while Nude mice inoculated with LevRad remained alive for 30 days.

Combined therapy of immunotherapeutic and antifungal agents has been tested as an adjuvant in potentializing the chemotherapy. This is an attempt to reduce toxicity of conventional drugs, the period of treatment and to prevent relapses of the disease [5]. Here we reported, for the first time, the immunotherapeutic potential of radioattenuated fungi associated with fluconazole in the treatment of PCM.

The immunization of infected BALB/c mice with LevRad associated with fluconazole (InRadMed) elicited a great therapeutic effect against highly infective yeast strains of *P. brasiliensis*. A reduction of CFU in the lungs from the InRadMed group in relation to animals only infected (C+) was already observed 60 days after infection. There was no fungal dissemination to the liver or the spleen. No fungal colonies were recovered from any organs (lung, liver and spleen) at 120 days post infection. It is safe to report that the combined administration of LevRad and fluconazole was capable of preventing relapse effects in this study. This was also confirmed by the histopathological analysis. Such analysis showed that the organs (lung, liver and spleen) preserved lung parenchyma as well as did not present yeast cells or granulomatous lesions. It is already well documented that resistance to *P. brasiliensis* infection is determined mainly by the host's ability to restrict fungal dissemination rather than the control of fungal growth in the primary infection site [37]. Moreover, it is important to consider the elimination of possible fungal quiescent focus as demonstrated 120 days after infection. The fungal cells may establish a dormant state and spontaneously reactivate. This reactivation may also occur when the host's immune system is impaired.

At the same time, the groups of infected animals immunized with LevRad (InRad) and treated with fluconazole (InMed) presented a significant reduction in the number of CFU, when compared to C+. This reduction was not observed between the InRad and the InMed groups though. The lungs obtained from the InMed and InRad groups presented organized well discrete focal neutrofilic and linfocytic granulomatous lesions with a few number of yeast cells. Immunization of infected animals with LevRad was effective, although at first it did not present benefits when compared with fluconazole treatment. However, it is important to highlight that the infected animals were immunized only twice with LevRad (no adjuvants), with a fortnight interval between events. On the other hand, fluconazole was administered for 30 days with no interruptions. Besides, animals from the InRad group were first evaluated 15 days after the medication was suspended whereas the animals from the InMed were evaluated immediately after the end of treatment.

The infected mice immunized with LevRad presented early expression (60 days after infection) of high TNF- $\alpha$  transcript levels in relation to the other groups (C+, InMed and InRadMed) which were not enough to ensure the fungi elimination. Moreover, it is plausible to believe that IL-4 may have contributed to the colonization and reproduction of the fungus in the lung, as well as its dissemination to the other organs (liver and spleen) as observed at 60 days post infection in InRad, InMed and C+. In contrast, we observed at 120 days after infection high transcript levels of IFN- $\gamma$  and iNOs enzyme especially in the InRad and InRadMed groups, with low IL-10 and IL-4 levels. This observation occurred concomitant with a complete resolution of the disease in InRadMed (120 days after infection). A significant decrease in the number of CFU and histopathological lesions was also observed in InMed and InRad. It is well known that IFN- $\gamma$  plays an important role in resistance to *P. brasiliensis* infection due to increase in the clearance of fungal cells, its regulation by cell-mediated immune responses and its regulatory effects on specific humoral immune responses [38]. NO may be considered a potent microbicide factor and its secretion is related with the fungus elimination and consequent host protection [39-41]. Regarding TGF- $\beta$ , the high expression verified at 120 days post infection may be due to the regulation of inflammatory response induced by proinflammatory cytokines [7,42,43].

In the present study, we also evaluated the specific antibody levels. High levels of IgG in the InMed, InRad and InRadMed groups were observed due to a response to the presence of the exoantigen of *P. brasiliensis* (Mexo). This suggests that this antigen may also contain B cell epitopes. A significant production of IgG1 was observed in all groups (C+, InMed, InRad and InRadMed) from 60 to 120 days of infection. High production of IgG2a, IgG2b and IgG3 in the InRad group was registered, as well as of IgG2b and IgG3 in the InRadMed group, after 60 days of infection. At 120 days, IgG2a and IgG2b levels were high in the InRad, InMed, InRadMed and C+ groups. However, IgG2a titers were higher in infected mice (immunized and treated) than in the C+ group. Protection against PCM has been attributed to a vigorous cellular immune response especially associated with Th1, whereas the high IgG levels and depressed cellular immune response was associated with progressive disease. Recently, however, some evidences have demonstrated the antibody-mediated protection against pathogenic fungi, such as antibodies to *Cryptococcus neoformans* polysaccharides or to *Candida albicans* cell wall components [44-46] as well as anti-gp70 Mabs [47] and some anti-gp43 Mabs [48] in experimental PCM. Besides, some IgG isotypes are associated with a Th1 response profile as well as works effectively on opsonization and activation of the complement system. IFN- $\gamma$  is considered the major inducer of a switch to IgG2a secretion, whereas IL-4 has been associated with isotypes switching to IgG1 and TGF- $\beta$  functions as an important Ig2b switch factor [11,49].

The high expression of IFN- $\gamma$ , iNOS and TGF- $\beta$ , and low expression of IL-4 and IL-10 (observed 120 days after infection) associated with high production of IgG2a and IgG2b, as well as, the low fungal load, the preserved tissues and low mortality rates suggest the predominant establishment of Th1-type immune response observed in InRadMed.

Finally, the results obtained in the present study provide important information concerning fungal immunotherapy demonstrating, by using yeast attenuated cells, that it is achievable for PCM. It was observed an additive effect of LevRad immunization to chemotherapy in PCM experimental that could be relevant mainly for studies dealing with drug resistance.

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